COMMENTS ON THE PROPOSED LISTING OF "NICKEL AND NICKEL COMPOUNDS" AS A REPRODUCTIVE OR DEVELOPMENTAL TOXICANT UNDER PROPOSITION 65

Comments of NiPERA, Inc.

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1. Executive Summary

In July 2018, California's Office of Environmental Health Hazard Assessment (OEHHA) issued a hazard identification document entitled "Evidence on the Developmental and Reproductive Toxicity of Nickel and Nickel Compounds." This document describes the evidence of developmental and reproductive toxicity of nickel and nickel compounds that will be considered by the Developmental and Reproductive Toxicant Identification Committee (DARTIC) in October 2018 when deciding on the listing of these substances as developmental or reproductive toxicant under Proposition 65. In response to this proposal, NiPERA Inc[1] wants to draw the DARTIC's attention to the following issues:

- **Nickel metal (*i.e.*, elemental nickel) needs to be considered separately from nickel compounds.** Chemically, they are totally different forms of nickel. The current Prop65 carcinogenicity group listing nickel metal and nickel compounds is based on NTP and IARC listings (1990). Since 1990, new data have been published that have not shown a significant association between increased lung cancer risk and exposure to metallic nickel. This listing should be reconsidered in the future.
- Furthermore, for systemic effects (like reproductive effects) that have thresholds and rely on systemic absorption, the evidence for **soluble nickel compounds** (highest bioavailability) needs to be considered separately from that of **insoluble nickel compounds** (very low bioavailability) and **nickel metal** (very low bioavailability and mostly present in alloys that are not ingested or inhaled).
    - ***When voting on whether nickel and nickel compounds "have been clearly shown through scientifically valid testing according to generally accepted principles to cause reproductive toxicity" we urge DARTIC to vote on the listings for soluble nickel compounds, insoluble nickel compounds, and metallic nickel, separately***

Developmental Effects:

- **Human evidence.** Studies of developmental effects such as spontaneous abortions (most closely related to the effects observed in rodents) did not demonstrate strong evidence of an association with nickel exposure. Likewise, the larger number of studies examining birth defects do not support a causal association between nickel exposure and birth defects. Some of the general public studies examining low birth weights report associations with air nickel. For the general public, the majority of the internal nickel dose is coming from naturally occurring nickel in the diet. Analyses comparing low birth weight results from highly exposed female nickel workers to those from minimally exposed public, demonstrate that the **statistical associations found in some of the general public studies (soluble and oxidic nickel exposures) were not realized in workers (exposed to all forms of nickel). In summary, these studies do not provide**

[1] NiPERA is the science branch of the Nickel Institute, the trade association of the world's major nickel producers. NiPERA sponsors research and performs evaluations of potential health and environmental risks and hazards associated with exposure to metallic nickel and nickel compounds.

evidence of causal associations between nickel and nickel compound exposure and developmental outcomes.

- **Animal evidence.** Reproductive toxicity listing of **soluble nickel compounds** (*e.g.*, sulfate, chloride, hydroxycarbonate) based on rat developmental effects **is warranted**; these effects are the most sensitive ones yielding the lowest point of departure (NOAEL or BMD). While there are no reliable animal reproductive studies with **water insoluble nickel compounds** by relevant routes of exposure, toxicokinetic studies indicate much lower systemic bioavailability. These data were considered by the EU Classification and Labelling subcommittee in 2008 and resulted in neither insoluble compounds nor nickel metal being listed for reproductive developmental effects even when the soluble compounds were. A single rat study with nickel metal nanoparticles reported developmental effects but the results are sparsely reported and there are issues regarding the statistical analyses conducted; this study does not provide sufficient evidence for listing nanoparticles, let alone other physical forms of **nickel metal**. There are no studies examining the reproductive effects of nickel-containing alloys. However, OEHHA should exempt nickel in alloys from Prop 65 listing for reproductive toxicity just as they have for Prop 65 carcinogenicity listing. It is difficult to imagine a scenario where increased blood levels of Ni could result from oral or inhalation exposure to alloys in coils or rods.
- **Weight of evidence.** The developmental effects seen with soluble nickel compounds in rodent studies have not been reproduced in human studies of the most highly nickel exposed workers. One possibility is that the mode of action of developmental toxicity in rodents is not relevant to humans. Another possibility, based on a comparison of internal doses (urinary nickel levels) between rats (at lowest LOAEC for developmental effects) and humans, indicate that the internal doses at which effects are seen in rats cannot be achieved in humans.

Female Reproductive Toxicity Effects:

- **Human evidence.** The general population studies examining fertility and other female reproductive effects are limited. Two of three studies reported null associations with the third study reporting associations for a subset of clinical chemistry parameters (not confirmed in other studies). **Thus, the overall human evidence does not support causal associations with nickel compounds exposure and is not sufficient for hazard listing.**
- **Animal evidence.** The listing of nickel metal and nickel compounds (soluble or insoluble) for female fertility effects is not supported by large and robust rat generational studies with the most bioavailable of the nickel substances (soluble compounds). **These studies have not shown soluble nickel compounds to affect female fertility even at doses above those that cause developmental effects.**

Male Reproductive Toxicity Effects:

- **Human evidence.** Of the 8 studies evaluating associations between nickel exposure and various male reproductive endpoints, 4 examined sperm functional parameters. Of these studies, 2 found effects on sperm motility and 2 did not; 3 of the 4 studies found no association with sperm counts. Urinary levels measured in 5 of 8 studies were within normal ranges; sperm

nickel levels were 8-fold higher in a study that did not show associations than in one that did. All the studies had limitations and moderate degree of risk of bias and **do not support a causal association between exposure to nickel compounds and male reproductive toxicity.**

- **Animal evidence**. The evidence for male fertility effects is conflicting but the more robust studies do not show adverse effects. **Thus, the listing of nickel and nickel compounds (soluble or insoluble) for male fertility effects is not warranted.**

Table 1 below summarizes NiPERA's views on the listing of nickel substances based on whether they meet the criterion of "*has been clearly shown through scientifically valid testing according to generally accepted principles to cause reproductive toxicity*"

	Water soluble and sparingly soluble Ni compounds	**Water insoluble Ni (oxidic & sulfidic) compounds**	**Ni metal**
Developmental Effects	Listing warranted based on animal studies by relevant route (it is the most sensitive reproductive endpoint, lowest POD). Relevance of results to humans is unclear. No listing warranted based on human studies (no causal association)	No listing warranted: no studies by relevant route but very low bioavailability based on toxicokinetic data No listing warranted based on human studies	No listing warranted based on animal studies by relevant route: very low bioavailability based on toxicokinetic data No listing warranted based on human studies
Female fertility Effects	No listing warranted based on animal studies by relevant route No listing warranted based on human studies	No listing warranted: no studies by relevant route but very low bioavailability based on toxicokinetic data No listing warranted based on human studies	No listing warranted based on animal studies by relevant route, very low bioavailability based on toxicokinetic data No listing warranted based on human studies
Male fertility Effects	No listing warranted based on animal studies by relevant route No listing warranted based on human studies	No listing warranted: no studies by relevant route but very low bioavailability based on toxicokinetic data No listing warranted based on human studies	No listing warranted based on animal studies by relevant route, very low bioavailability based on toxicokinetic data No listing warranted based on human studies

2. Introduction

NiPERA Inc. appreciates the opportunity to provide comments on the evidence presented in the OEHHA hazard identification document describing the developmental and reproductive toxicity data for nickel and nickel compounds. This is a very comprehensive and well laid out document.

However, the quality of the animal and human studies and the power to detect reproductive effects varies widely. Thus, the identification of reliable studies and the interpretation and significance of results requires a systematic review, which was not conducted in this case. In addition, the consistency of the data, taking into account the quality and relevance of positive and negative findings, needs to be considered using a weight of evidence approach; the overall assessments should not be based mainly on the studies with positive findings regardless of study reliability (see comments submitted by Gradient on behalf of NiPERA). The population-based studies looking at associations between nickel air exposures and reproductive outcomes often suffer from limitations derived from their reliance on single pollutant models to assess risks in multi-pollutant studies. While these studies are useful to generate hypotheses, they are not robust enough to establish reliable evidence of causality (see comments submitted by S. Seilkop on behalf of NiPERA).

With regards to the animal studies, we focus our comments on those conducted by relevant routes of exposure (oral, inhalation, dermal). When examining positive results in injection studies with nickel compounds the following should be considered: doses achieved via injection are usually much higher than those that can be achieved via oral or inhalation routes. On average, only 10% of an oral dose of the most bioavailable of the nickel compounds is absorbed systemically. Furthermore, as nickel is absorbed from the gastrointestinal tract it binds to transport proteins in blood. This physiological mechanism is overwhelmed when free nickel ion is given by injection.

Our comments address some issues that we think are of high relevance for DARTIC and complement the comment on the human evidence independently submitted by Drs. J. Goodman and R. Pruitt of Gradient; the ones on low birth weight studies independently submitted by S. Seilkop of SKS Consulting Services; as well as those on the animal evidence for female and male reproductive effects by relevant routes of exposure provided by Drs. J. DeSesso and A. Lavin Williams of Exponent included with these NiPERA comments (Attachment 1).

3. Chemical information about nickel metal and nickel compounds

The potential listing of nickel metal needs to be considered separately from that of nickel compounds. Chemically, they are totally different forms of nickel: nickel metal has zero valence and requires surface corrosion to release Ni(II) ions, while nickel compounds have typically valence II and release Ni ion through dissolution. Nickel metal (*i.e.*, elemental nickel) is not a nickel compound, and alloys containing nickel metal (zero valence) are also not nickel compounds. Alloys are special mixtures where more than one element (metal or metalloid) are combined in ways that cannot be separated by physical means. The synonyms given to nickel metal in Table A1 of the OEHHA document include the names of many alloys where nickel is just one of several components; most of these alloys are present as sheets, coils or

rods. These forms of nickel metal and alloys are not relevant to the evaluation of reproductive toxicity as they cannot be eaten or inhaled and will not affect the internal nickel doses which are mainly driven by daily consumption of food and water[2].

Whether by inhalation or via oral route, the systemic absorption of nickel from soluble nickel compounds is greater than for insoluble compounds or nickel metal. For this reason, the **bioavailability and systemic toxicity of soluble nickel salts are much greater than that of insoluble compounds or nickel metal**. This is evident by:

1. The results of Ishimatsu *et al*. (1995) study that are reported under the Pharmacokinetics section of the OEHHA document. Rats orally exposed to the same amount of different chemical forms of nickel demonstrated that exposure to nickel salts resulted in the highest nickel absorption and bioavailability. In particular, there was a ≥100-fold lower oral absorption of nickel from nickel oxide and nickel metal powder compared to nickel salts.
2. Acute oral toxicity studies showing much lower LD_{50} values for nickel salts (*i.e.*, higher toxicity) than for insoluble nickel compounds and nickel metal, indicating lower (systemic) Ni ion bioavailability.
3. Bioelution studies in fluids relevant to the oral route (*e.g.*, surrogate gastric fluid) that show significant differences in bioaccessible[3] Ni ion in these fluids. Bioaccessibility of nickel in oral fluids provides a conservative estimate of bioavailability since less than a third of the bioaccessible ions are absorbed as they move through the gastrointestinal tract.

These results are summarized in Table 2 below.

Since reproductive effects are systemic effects associated with the bioavailable Ni(II) ion, it is important to understand the systemic absorption of nickel from the chemical forms of nickel present in various media (air, water, diet, etc.) through their relevant routes of exposure.

Oral Route: The oral route of exposure is relevant to the ingestion of nickel from water, foods, soils, and in the case of the animal studies, administered via gavage or with feed or drinking water. In addition, there is a fraction of the inhaled particles (from ambient air or workplace air) that deposits in the extra-thoracic (nose) and trachea-bronchial (TB, conducting airways) regions of the respiratory tract and is absorbed via the gastrointestinal tract.

[2] In a May 7th, 2004 Notice to Interested Parties, OEHHA clarified that nickel alloys are not nickel compounds and are exempt from carcinogenicity listing under Prop 65: "For the purposes of clarification, OEHHA notes that nickel alloys are distinct from nickel compounds, and are not included in the Proposition 65 listing of nickel compounds." The Notice goes on to say "A nickel alloy is a mixture of nickel with one or more other elements, typically produced by mixing molten nickel with other substances. The atoms in an alloy are not covalently or ionically bonded in fixed ratios."

[3] Bioaccessibility of a metal from a substance or mixture can be estimated in vitro by measuring the quantity of a metal ion released under physiological conditions in bioelution tests with surrogate biofluids.

Table 2. Oral bioavailability and acute toxicity

Test Substance	Gastric Bioaccessibility- 2 hours (% Ni released)[a]	Intestinal Bioaccessibility 24 hours (% Ni released)[a]	Absorbed Fraction 24 hours (%)[b]	Acute Toxicity (oral LD_{50}; mg Ni/kg/bw)[c]
Ni sulfate hexahydrate	90.55	58.00	11.12	83
Ni chloride hexahydrate	89.85	38.45	9.8	125
Ni hydroxycarbonate	84.30	1.5	NA	980
Ni oxide (black)	29.60	0.32	0.04	7500
Ni subsulphide	22.65	0.25	0.47	>7700
Ni sulphide	9.75	0.18	2.12	NA
Ni oxide (green)	0.33	0.12	0.01	>8900
Ni metal	18.2	NA	0.09	>9000[d]

NA, not available.

a. Bioaccessibility reported as the percent of available Ni content released in synthetic gastric fluid after 2 hours or intestinal fluid after 24 hours. Reported values are mean values from duplicate experiments. Henderson *et al.* (2012 a).

b. Ishimatsu *et al* (1995).

c. Henderson *et al.* (2012 b).

d. FDRL (1983).

Nickel (II) in gastric fluid is ~100% soluble (100% bioaccessible), but its absorption is modulated by the presence of food. Oral absorption of dissolved nickel ion from water ranges from 1-5% (when ingested with food) to 12-27% (when ingested under fasting) in studies of human volunteers (*e.g.*, Nielsen *et al*., 1999). The gastrointestinal absorption of nickel (II) from nickel naturally present in food is low (1-5%) since nickel is present in plants as complex organic molecules and is not easily bioaccessible in gastric or intestinal fluids (Olivares Arias *et al*., 2015).

The oral absorption of insoluble nickel compounds is much lower than that of water soluble nickel compounds as described in rat toxicokinetic and acute toxicity studies and predicted by their much lower bioaccessibility in gastric fluids (Ishimatsu *et al*., 1997; Henderson *et al*., 2012a, 2012b, Table 2).

Dermal route: Systemic absorption of nickel ion via the dermal route is very low. Dermal absorption is estimated at ~2% for water-soluble nickel compounds (Tanojo *et al*., 2001) and 0.2% for the metallic form (Hostynek *et al*., 2001).

Inhalation route: In ambient air, nickel is mainly present as nickel sulfate (water soluble) and complex nickel oxides (*e.g.*, US EPA, 1986; ATSDR, 2005). By contrast, nickel refinery workers are exposed to a mixture of water soluble and insoluble compounds and metallic nickel (*e.g.*, Thomassen *et al*., 1999). Welders of alloyed materials (*e.g.*, steel) are exposed to mixtures of gases and particles where nickel is present as very fine complex nickel oxides (*e.g.*, spinels, IARC 2018). Systemic absorption of nickel via inhalation will depend on the particle size and respiratory tract deposition of the aerosol as well as on the bioaccessibility of the nickel substance in lung fluids. Larger particles will be deposited in the upper respiratory tract (head and TB regions), swallowed, and a small fraction absorbed from the

gastrointestinal tract. Smaller particles of water-soluble nickel compounds that are deposited in the pulmonary region of the respiratory tract are assumed to be highly dissolved and absorbed, while insoluble particles will be absorbed to a much lower extent and will eventually be cleared to the gastrointestinal tract.

There is a good correlation between inhalation exposure to water-soluble aerosols and blood and urine nickel levels in humans (*e.g.*, Thomassen *et al*. 1999). There is also a good correlation between oral exposures to water soluble nickel and blood and urine nickel levels in rats (Heim *et al*., 2007). It should be noted, however, that the internal exposures achievable in the rat gavage studies are not achievable in humans, even in the most highly exposed workers. See further discussion on this point under Section 5. Developmental toxicity.

4. Separate consideration of nickel metal, soluble, and insoluble nickel compounds

Inhalation of high levels of aerosols containing mixtures of water soluble, sulfidic and oxidic nickel compounds present during the sulfidic ore production and refining of nickel has been associated with increased risk of respiratory tumors in epidemiological studies (*e.g.*, ICNCM, 1990). Studies by inhalation and ingestion in animals confirmed that these compounds can only cause tumors via inhalation (local respiratory tract tumors) and not via oral exposure (Dunnick *et al*., 1995; Heim *et al*., 2007).

While Prop 65 (1989) lists nickel metal and compounds as known to the State of California to cause cancer, the IARC and NTP carcinogenicity listings make a distinction between them.

- IARC (1990) listing: Group 1 (nickel compounds), **Group 2B (nickel metal**, suspect carcinogen)
- NTP 10th RoC (2000) listing: known to be human carcinogens (nickel compounds); **reasonably anticipated to be human carcinogen (nickel metal);** not listed (nickel alloys)
- In Europe, nickel compounds are classified as Category 1A carcinogens, while **nickel metal is classified as Category 2** (suspect carcinogen) under the CLP Regulation.

In 2005, a review of the epidemiological evidence for the carcinogenicity of nickel metal confirmed the lack of an association between metallic nickel exposures and increased respiratory cancer risks in workers (Sivulka *et al.,* 2005); this is the same finding reported in the 1990 seminal epidemiological study where respiratory cancer risk of 80,000 workers in primary nickel production and processing of nickel alloys was studied (ICNCM, 1990). Consistent with these findings, in 2008, a rat carcinogenicity study with nickel metal powder by the relevant route of exposure (inhalation) showed no increased risk of lung tumors (Oller *et al*., 2008).

Based on the latest epidemiological and animal data, it does not appear that nickel metal should be listed as a carcinogen in Prop 65. This listing should be reconsidered in the future.

We encourage DARTIC to not base a grouping for reproductive toxicity on the grouping for cancer. For systemic effects that have thresholds and rely on systemic absorption (*e.g.*, reproductive effects), the evidence for soluble nickel compounds (highest bioavailability) needs to be considered separately from that of insoluble compounds (lowest bioavailability) and nickel metal (low bioavailability and mostly found in alloys that are not ingested or inhaled).

> ***When voting on whether nickel substances "have been clearly shown through scientifically valid testing according to generally accepted principles to cause reproductive toxicity" we urge DARTIC to vote on the listings for soluble nickel compounds, insoluble nickel compounds, and metallic nickel, separately.***

5. Developmental toxicity

Human evidence.

Adverse pregnancy outcomes. Three of four studies investigating the association between spontaneous abortions and premature births with nickel exposures did not report any associations (Vaktskjold *et al*., 2008a; Zheng *et al*., 2014; Manduca *et al*., 2014). One study (Chashschin *et al*., 1994) did. However, the early study by Chashschin *et al*. was superseded by the studies by Vaktskjold *et al*. (of which Chashschin is a co-author)[4]. The latter, more robust study did not reproduce the earlier findings.

Birth defects. Seven studies evaluated the statistical associations between nickel exposure and birth defects. Of these, 2 studies reported positive associations (Chashschin *et al*., 1994; Zheng *et al*., 2012) and five did not (Friel *et al*., 2005; Vaktskjold *et al*., 2006; 2008b; Manduca *et al*., 2014; Yan *et al*., 2017). As mentioned above, the studies by Vaktskjold *et al*. updated the earlier Chashschin *et al*. study. Considering that most of the studies are negative (including those with more reliable results), the evidence does not support a causal association between exposure to nickel and nickel compounds and birth defects.

Low birth weights. Ten studies evaluated the statistical associations between nickel exposure and low birth weight (LBW). Four studies reported positive associations (Bell *et al*., 2010; Ebisu and Bell., 2012; Basu *et al*., 2014; Laurent *et al*., 2014) and one reported on a borderline effect (Pederson *et al*., 2016). Five of ten studies reported no or negative associations (Odland *et al*., 1999, 2004; McDermott *et al*., 2014; Hu *et al*., 2015; Vaktskjold *et al*., 2007). The Bell *et al*. (2010), Ebisu and Bell. (2012); and Vaktskjold *et al*. (2007) studies were considered to have the lowest risk of bias (see Gradient Comments submitted separately). Analyses comparing results from the highly exposed workers' study of Vaktskjold *et al*. (2007) to those from minimally exposed public (Ebisu and Bell, 2012), demonstrate that the statistical associations found in some of the general population studies were not realized in workers.

[4] As a consequence of very preliminary observations reported by Chashschin *et al.* (1994), a retrospective series of analyses of reproductive outcomes among employees at a nickel refinery started in 1995. Personal exposure and biomonitoring data were collected and a birth registry was established. These studies were co-funded by the Monchegorsk Municipal Environmental Fund, the Norwegian Ministry of Health through the Barents Health Co-operation Program, the Norwegian Research Council, NiPERA, and the Ontario Workplace Safety and Insurance Board (WSIB).

For example, the odds ratio for low birth weight predicted from the work of Ebisu and Bell (2012) were not realized in the refinery workers' studies (Vaktskjold *et al*., 2007) with daily air nickel absorption levels 60 to >350-fold higher than the minimal needed to detect these effects with sufficient statistical power (see S. Seilkop comments submitted separately). Thus, these studies do not provide evidence of causal association with nickel and nickel compounds.

Other endpoints. Studies looking at endpoints like Autism Spectrum Disorders, retinoblastoma, oxidative damage do not provide sufficient evidence of a causal association.

Animal evidence.

Soluble Nickel Compounds: As noted in the OEHHA document, there are several reliable studies of the developmental effects of water-soluble nickel compounds in rats and mice and these studies have demonstrated adverse developmental effects. In 2008, the European Commission (EC) reviewed the available animal and human data on the developmental toxicity of nickel substances and concluded that there was enough evidence from animal studies to assign a harmonized classification of animal reproductive toxicant (Cat 1B) developmental effects to the readily water-soluble nickel compounds such as nickel sulfate and nickel chloride (Annex VI; CLP Regulation, 2009; EURAR, 2008-2009b,c). This classification applied specifically for developmental effects (*i.e.,* perinatal mortality) observed in large, well-conducted studies on rats. These studies demonstrated that absorption of nickel ion into systemic blood circulation after high oral exposure (*e.g.* LOAEL of 2.2 mg Ni/kg bw/d) to water-soluble nickel compounds during pregnancy increased the death rate of the offspring around parturition (*e.g.*, Smith *et al.*, 1993; Siglin, 2000a,b). While no other developmental effects, including malformations (*i.e.,* teratogenesis), were identified in a rat prenatal developmental toxicity study with water-soluble nickel chloride at the maximum tolerated dose of 42 mg Ni /kg bw/day (RTI, 1988a,b, sometimes cited as Price, 1988 in OEHHA document), nickel chloride was shown to cause malformations (*e.g.* microphthalmia) in a prenatal developmental toxicity study in mice at 46 mg/kg bw/day and other teratogenic effects at higher doses (Saini *et al*., 2013). Based on these studies, **the Prop 65 listing of soluble nickel compounds based on rodent developmental effects is warranted, with the most sensitive effect being perinatal mortality.**

Insoluble Nickel Compounds and Nickel Metal. While no reliable animal studies are available assessing the developmental toxicity of insoluble nickel compounds or nickel metal, the available evidence indicates that they would not be expected to cause developmental toxicity. In 2008, the EC determined that insoluble nickel compounds, such as nickel oxides and sulfides, and also metallic nickel, did not meet the criteria to be classified as reproductive toxicants (EURAR, 2008-2009a,b). This was based on toxicokinetic data indicating the much-reduced oral bioavailability of Ni ion from these substances compared to soluble nickel compound exposure in rats (Ishimatsu *et al*., 1995). In the case of nickel metal, 100-fold lower oral absorption was observed compared to nickel chloride or nickel sulfate. Very high LD_{50} and LC_{50} values (Table 2) demonstrating low acute oral and inhalation toxicity for nickel metal and insoluble nickel compounds compared to soluble nickel compounds are consistent with the significantly lower absorption of Ni ions from these substances.

The study of Kong *et al*. (2014) examined potential reproductive effects (including developmental toxicity) associated with oral exposure to nickel nanoparticles. However, this study suffers from methodological and statistical shortcomings. While increased perinatal mortality was observed in the treatment groups, control pups had a survival rate at weaning of only 79%. Some of the reported changes in females may be related to a non-treatment-related increase in the number of animals undergoing proestrus at the time of sacrifice, while the reported histological changes in the testes could be artifacts of incomplete fixation (the wrong fixative, paraformaldehyde, was used for that tissue). For more information on the shortcomings of this study, please see the Exponent review included as Attachment 1 to these comments. The data regarding other forms of nickel metal in the study were also insufficient for listing.

When all the data are considered in a weight of evidence approach, **the data do not support nickel metal or insoluble nickel compounds causing developmental toxicity effects and therefore the criteria for Prop 65 listing for these nickel substances is not met.** A separate analysis of these effects by Exponent (Attachment 1) agrees with these conclusions for insoluble nickel compounds and nickel metal. It is important to note that with regards to nickel metal, the European Chemicals Authority (ECHA) is currently evaluating a testing proposal submitted by Vale, Inc., to conduct an extended one generation reproductive toxicity study (EOGRTS) with nickel metal micron size powder via the oral route to fulfill REACH testing requirements. It would be premature to list nickel metal on Prop 65 for reproductive or developmental toxicity before this definitive study is complete.

Combined animal and human evidence.

As indicated above, there are multiple rat studies with soluble nickel compounds (including the NiPERA-sponsored reproductive studies, Siglin, 2000a,b) showing increased perinatal mortality at daily oral exposures above 1.1 mg Ni/kg b.w. (administered by gavage). The mode of action for these effects is not currently known. Studies of female workers exposed to high levels of water soluble nickel compounds via inhalation failed to show an association between exposures to nickel and observed adverse reproductive effects (Vaktskjold *et al*., 2006; 2007; 2008a; 2008b).

To place the animal and human results in context, one can compare the urine nickel levels in the workers' cohort with the urine levels in the rat studies. Table 3 lists the workplace nickel exposures relevant to the results reported in the Vaktskjold *et al*. studies.

Table 3. Urine nickel values (from Vaktskjold *et al.,* 2006 unless otherwise noted).

	GM Urine Ni µg Ni/L	Mean urine Ni µg Ni/L	Upper 95% CI urine µg Ni/L
Control			
-background[1]	5.9		7
-sulfuric acid production	6.3		8.4
Mean	**6**		**7.7**
Low exposure			
-copper electrorefining	15		18
-Cu pyrometallurgical	8.3		10
-matte converting	20		32
-beneficiation	5.1		6.6
-ore roasting	11		14
-ore smelting	20		26
-matte separation	29		41
Mean	**15.5**		**21**
High exposure			
-anode casting old	159[2]	268[2]	208
-anode casting new	131[2]	337[2]	186
-electrorefinery old	179[2]	293[2]	230
-electrorefinery new	127[2]	191[2]	169
-matte roasting	87[2]	121[2]	106
-Ni carbonyl plant	47	NA	81
Mean	**122**	NA	**163**

GM: geometric mean; CI: confidence interval

1. From Odland *et al*. (1999)

2. Values from Thomassen *et al*. (1999).

Background urinary nickel levels in non-refinery females had a geometric mean of 5.9 µg Ni/l, this value is at the high end of background levels for unexposed populations. The low exposure refinery workers had a geometric mean of 15.5 µg Ni/l with a P95 of 21 µg Ni/l (~3-fold increase in urinary levels) and the high exposure workers had a geometric mean of 122 µg Ni/l with a P95 of 163 µg Ni/l (20-fold increase in urinary levels). Urinary levels of 70 µg Ni/l were chosen as the cutoff between low and high exposure workers.

In a rat oral chronic study with nickel sulfate (highest bioavailability), rats were exposed to 2.2 to 10 mg Ni/kg and blood and urinary nickel levels were measured after a two-year exposure (Heim *et al*., 2007; Rush, 2005). A linear dose-response between oral intake of nickel and urinary nickel levels was found. At the lowest exposure of 2.2 mg Ni/kg, the mean urine value was 2300 µg Ni/L (males + females). The exposure level of 2.2 mg Ni/kg corresponds to the LOAEL for perinatal mortality effects in a robust rat study (Siglin, 2000 a,b). The rat urinary nickel level corresponding to the LOAEL for reproductive effects is 150-fold and 20-fold higher than those measured in Low and High exposure nickel refinery workers, respectively.

This analysis strongly indicates that the reproductive effects observed in rats 1) may not be relevant to humans or 2) may not be achievable even in the highest exposed workers. In either case, the relevance

of the positive results in rats (at unachievable human exposures) needs to be considered together with the negative results in human studies for the highest exposed human population.

6. Female reproductive toxicity

Human evidence.

Three general population studies examining fertility effects associated with nickel exposure were included in OEHAH doc. Two of the 3 studies report null associations (Bloom *et al*., 2011; Maduray *et al*., 2017). The third study (Zheng *et al*., 2015) investigating associations between nickel exposures and PCOS, had positive findings for some clinical chemistry parameters (*e.g.*, SHBG) but not for some sex hormones like estradiol or testosterone. **Overall, the human evidence does not support a causal association with nickel compounds and is not considered sufficient for hazard listing.**

Animal evidence.

Ten studies conducted by oral exposure which examined hormone alterations, ovarian histology, effects on implantation, and perinatal pup mortality were identified in the OEHHA document. Potential female fertility impairment due to oral or inhalation exposure to nickel compounds (including soluble nickel compounds, which are the most bioavailable forms) has been extensively studied in reliable studies, and no effects on fertility have been found. There are several reliable 13 week-and one- and two-generation studies utilizing inhalation or oral administration of water-soluble nickel compounds in rats that have not indicated adverse effects on fertility, estrous cycling, vaginal cytology, copulation and fertility indices, precoital intervals, gestation lengths, gross necropsy findings and histopathology with doses up to 31.6 mg Ni/kg/day (Smith *et al*., 1993; RTI 1988a,b; NTP, 1996a,b,c; Siglin, 2000a,b). Because the reproductive toxicity effects are related to the bioavailable nickel ion, the lack of fertility effects following exposure to water-soluble nickel compounds (which have higher bioavailability) is relevant to water-insoluble nickel compounds and nickel metal, which have much lower bioavailability (Ishimatsu *et al*., 1995). Therefore, while water-soluble nickel compounds have been shown to cause developmental effects in rodents, neither water-soluble nickel compounds, nickel metal, nor insoluble nickel compounds have been shown to affect female fertility. Therefore, **neither soluble nickel compounds, insoluble nickel compounds, nor nickel metal have met the criteria for listing under Prop 65 as having been clearly shown through scientifically valid testing according to generally accepted principles to cause female reproductive toxicity.**

7. Male reproductive toxicity

Human evidence.

Of the eight studies included in OEHHA document evaluating associations between nickel exposure and various male reproductive endpoints, 4 examined sperm functional parameters. Sperm functional parameters are more direct indicators of adverse effects such as infertility than markers such as sperm DNA damage. Of these studies, 2 found effects on sperm motility (Danadevi *et al*., 2003 and Zafar *et al*.,

2015) and 2 did not (Skalnaya *et al*., 2015 and Zeng *et al*., 2015); 3 of the 4 studies found no association with sperm counts (Danadevi *et al*., 2003; Skalnaya *et al*., 2015; Zeng *et al*., 2015). Urinary levels measured in 5 of 8 studies were within normal 1-6 µg Ni/L ranges (Sancini *et al*., 2014; Zeng *et al*., 2013; 2015; Wang *et al*., 2016; Zhou *et al*., 2016); sperm Ni levels were 8-fold higher in a study that did not show associations (Skalnaya *et al*., 2015) than in one that did (Zafar *et al*., 2015). Given the inconsistencies of the results, the use of inappropriate statistical analyses in some of them, and the fact that several of the studies included infertile males or males undergoing fertility treatment**, the human evidence is not considered sufficient for listing of nickel compounds as male reproductive toxicants.**

Animal evidence.

There are ten studies with relevant routes of exposure that assessed potential male reproductive effects of concern including hormone alterations, effects on reproductive organ weights, testicular histopathology, alterations in sperm motility, and effects on fertility. For many of these parameters, the data are highly contradictory across studies. While some studies reported changes in testicular histopathology, many of the studies reporting possible effects are likely confounded by improper tissue fixation methods. This issue reduces the strength of these studies for drawing conclusions regarding male reproductive toxicity. Based on the conflicting nature of the findings reported and the methodological shortcomings regarding tissue fixation, the data are considered to be insufficient to warrant the listing of nickel as a male reproductive toxicant.

As with effects on female fertility discussed above, the Prop 65 listing of nickel or nickel compounds for male toxicity effects is not supported by large and robust rat generational studies (Smith *et al*., 1993; Siglin, 2000a,b) that have not shown nickel to affect male fertility, even at doses above those that cause developmental effects. There are several reliable 13 week and one- and two-generation studies utilizing inhalation or oral administration of water-soluble nickel compounds in rats that have not indicated adverse effects on sperm parameters, copulation and fertility indices, precoital intervals, gross necropsy findings and histopathology with doses up to 31.6 mg Ni/kg/day (Smith *et al*., 1993; RTI, 1988a,b; NTP, 1996a,b,c; Siglin, 2000a,b). While some male reproductive effects have been reported in studies in mice with soluble nickel (*e.g.* Pandey and Srivastava, 2000), they were inconsistent across studies and the European Commission (EC) reviewed these data and did not consider them sufficient to classify soluble nickel compounds for reproductive or fertility effects (EURAR, 2008-2009a,b). The review by Exponent found in Attachment 1 confirms the conclusion that no reliable evidence exists to indicate that nickel or nickel compounds cause male reproductive toxicity. Therefore, **neither soluble nickel compounds, insoluble nickel compounds, nor nickel metal have met the criteria for listing under Prop 65 as having been clearly shown through scientifically valid testing according to generally accepted principles to cause male reproductive toxicity.**

8. Conclusions

A thorough examination of the evidence of potential developmental and reproductive toxicity effects of nickel and nickel compounds indicates that **the only effects that have been clearly shown through scientifically valid testing according to generally accepted principles are the developmental toxicity effects observed in animal studies with soluble nickel compounds**. There is no clear evidence that nickel metal or insoluble nickel compounds cause developmental toxicity effects, and there is no clear evidence that nickel metal or soluble or insoluble nickel compounds cause any male or female reproductive effects. Furthermore, while statistical associations between developmental effects and nickel exposure have been purported in some large general public studies, the effects have not been demonstrated in workers' studies with much higher air exposure levels and the power to detect these effects. Therefore, only soluble nickel compounds should be listed under Prop 65, and only for developmental effects based on animal evidence. **Nickel metal and insoluble nickel compounds should not be listed for developmental or female or male reproductive effects.**

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10. Attachment 1: Exponent's Evaluation.

Exponent®

Evaluation of the Reproductive Toxicity Database for Nickel

Evaluation of the Reproductive Toxicity Database for Nickel

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Background and Purpose

The California Office of Environmental Health Hazard Assessment (OEHHA) is in the process of reviewing the developmental and reproductive toxicity (DART) data for nickel and nickel compounds under the authority of the California Safe Drinking Water and Toxic Enforcement Act of 1986, commonly referred to as "Proposition 65". This review is being conducted by OEHHA's Developmental and Reproductive Toxicant Identification Committee (DARTIC). Exponent scientists were requested by NiPERA, Inc. to review a select set of animal studies involving oral exposure to nickel or nickel compounds in order to provide an opinion on the strength of the data with particular focus on female and male reproductive effects. The 15 papers for evaluation (with some overlap between female and male reproductive effects across studies) are as follows.

Studies addressing female reproductive effects (10 studies)

1. Käkelä R, A Käkelä, H Hyvärinen. 1999. Effects of nickel chloride on reproduction of the rat and possible antagonist role of selenium. Comp. Biochem. Physiol., Part C 123:27-37.
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Studies addressing male reproductive effects (10 studies)

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This assessment of the available data for oral nickel exposure to animals is being done with specific consideration of the criteria that have been established by DARTIC for determining whether the weight-of-evidence is sufficient for listing a given compound as a reproductive toxicant (OEHHA, 1993). For the purposes of completing this analysis, we relied primary on the select reproductive toxicity studies of nickel and nickel compounds provided to us by the client, as well as tabulated information provided in the OEHHA Report on Evidence on the Developmental and Reproductive Toxicity of Nickel and Nickel Compounds (OEHHA, 2018), information available in the peer-reviewed scientific literature, textbooks of reproductive biology, and our combined expertise (>60 years) in the areas of developmental and reproductive toxicology.

Select Reproductive Toxicity Database for Nickel

Because it is the most relevant route of exposure for humans, NiPERA, Inc. identified a select set of 15 studies involving oral exposure of animals to nickel and nickel compounds for evaluation; these are listed in Tables 1 and 2. In general, these studies are of limited methodological and reporting quality. While some of the studies incorporated designs typically used to assess reproductive toxicity (for example, Smith et al., 1993; Käkelä et al., 1999; Siglin, 2000a,b), many of the studies evaluated herein used novel study designs more appropriate for mechanistic investigations than for a thorough evaluation of potential reproductive effects. Consequently, most of the studies listed in Tables 1 and 2 failed to incorporate good laboratory practices (GLP) and many used small group sizes (fewer than 10 animals per group). Also, while the majority of these studies incorporated multiple dose groups in their design, a few (Schroeder and Michener, 1971; Käkelä et al., 1999 for males only; Pandey and Singh, 2001; Toman et al., 2012) used only a single nickel treatment group.

Inconsistencies also exist across the body of studies with respect to the specific form of nickel administered. Kong et al. (2014) exposed rats to nickel metal nanoparticles and microparticles. All other studies involved administration of nickel salts: either nickel sulfate ($NiSO_4$) or nickel chloride ($NiCl_2$), which are aqueously soluble, or in one case, nickel carbonate ($NiCO_3$), which is insoluble in water. Nickel metal is generally not absorbed orally to any appreciable extent, while $NiSO_4$ and $NiCl_2$ are minimally absorbed (~10%) via oral administration (Ishimatsu et al., 1995). Based on what is known about other metal nanoparticles (Lin et al., 2015), nickel metal nanoparticles, although likely to be absorbed to a greater extent than non-nanoparticles, are still unlikely to be absorbed orally to the same extent as the soluble nickel compounds.

Sometimes, the test articles were reported as being the anhydrous material; other times, the study investigators indicated that they had used the hexahydrate form of the salt (e.g., $NiSO_4 \cdot 6H_2O$ or $NiCl_2 \cdot 6H_2O$). However, it often is not clear which form of the salt was administered due to the lack of detail provided in the study reports, leaving the reader to guess. This issue of inconsistency is further compounded because of disparities across studies in the units in which the doses were reported. In many of the drinking water studies, the doses are

simply reported in ppm or % of nickel in the drinking water; in these circumstances, the daily amount of water consumed by the animals and the animal's body weight must be considered in order to compare the doses received to those administered in other studies. In some cases, the doses were reported in terms of mg of *nickel*/kg body weight per day; in other cases, the doses are stated in terms of mg of *test material*/kg body weight per day. In an effort to address this issue and allow for comparisons across studies, Tables 1 and 2 we have converted all doses in the select studies to mg nickel/kg body weight per day.

Finally, the methods of administration varied across studies. Some of the studies involved drinking water, feed, or "pellet" administration, all of which generally provide for more sustained, but lower maximum, internal nickel concentrations than are achieved using gavage bolus dosing. These differences must be taken into consideration when attempting to compare the results observed across studies.

The following analysis will highlight the reported findings for this body of studies, any shortcomings in the various studies, and what the overall body of information is able to provide regarding the potential reproductive toxicity of oral exposure to mostly water-soluble nickel compounds.

Female Reproductive Toxicity

Ten (10) studies involving oral exposure of animals to nickel or nickel compounds and evaluation of potential female reproductive effects were reviewed; these are listed in Table 1. Of these, the studies with the designs of highest relevance for assessing female reproductive effects are considered to be Smith et al. (1993), Siglin (2000b) and Kong et al. (2014) based on the inclusion of multiple dose groups, large numbers of animals per group, and the incorporation of dosing regimens typically used for reproductive toxicity assessments. However, the execution of these studies has not been ideal. Kong et al. (2014) evaluated non-standard endpoints not typically assessed in reproductive toxicity evaluations and suffers from some methodological shortcomings, as discussed in detail below. Further, the statistical evaluations conducted in this study are questionable, as some of the findings reported to be significantly different from control vary little from control values; thus, the reported findings lack credibility.

Although Siglin (2000b) was conducted in a GLP laboratory using a study design commonly employed for the evaluation of reproductive toxicity, the doses administered in this study (up to 10 mg/kg/day of $NiSO_4 \cdot 6H_2O$) were likely too low. This conclusion is based on results of the preceding dose range-finding study (Siglin, 2000a), which reported effects on early pup survival and mean litter size at 75 mg/kg/day and increased post-implantation loss at ≥30 mg/kg/day. Because guidance indicates that the highest dose administered in a reproductive toxicity study is meant to "induce toxicity, but not death or severe suffering" (OECD, 2001), based on results of the dose range-finding study, the high dose in the definitive study should have been at least 30 mg/kg/day or more. Nevertheless, it must be recognized that Siglin (2000b) established a NOAEL for reproductive toxicity of at least 10 mg/kg/day (*i.e.*, 2.2 mg Ni/kg/day).

Table 1. Select studies of oral nickel exposure on female reproductive endpoints.

Reference	Species/strain (N)	Route	Test material	Reported doses/duration	Nickel doses (mg Ni/kg/ day)	Effects
Rats						
Kong et al., 2014	Rat (SD) (20/group)	gavage	Ni nano-particles (NP) or micro-particles (MP)	NP: 0, 5, 15, 45 mg/kg/day MP: 45 mg/kg/day 10 weeks prior to mating through lactation	0, 5, 15, 45	No effects on ovary relative weights, mating or fertility Ovary histopath (vascular dilation/congestion, lymphocytosis, ↑/cavitated luteal cells, ↑ eosinophils/inflammatory cells) ↑ FSH, LH; ↓ E2 ↓ Birth survival rate", "feeding survival rate", pup weights
Schroeder and Michener, 1971	Rat (Long Evans) (5/group)	drinking water	Ni-soluble salt (specific form not reported)	0, 5 ppm [a] Weaning to 9 mo of age and through 3 generations	0, 0.7 [b]	↑ Early pup deaths, # runts ↓ F3 litter size
Smith et al., 1993	Rat (Long Evans) (34/group)	drinking water	$NiCl_2$	0, 10, 50 250 ppm Ni (0, 1.3, 6.8, 31.6 mg/kg/day) 11 weeks prior to mating through 2 generations of animals	0, 1.3, 6.8, 31.6	No effect on indices of reproductive performance, # pups/litter, pup weights ↑ Dead pups at birth, early pup deaths (at 250 ppm in 1st gen, all doses in 2nd gen) ↓ Pup survival to weaning (at 250 ppm in 1st gen, at ≥50 ppm in 2nd gen) ↓ Prolactin levels in dams at 250 ppm
Käkelä et al., 1999	Rat (Wistar) (6/group)	drinking water	$NiCl_2 \cdot 6H_2O$	0, 10, 30, 100 ppm Ni [c] 2, 4 or 14 weeks prior to mating through lactation	0, 1.4, 4.2, 14 [b]	No effect on fertility, gestation length, # "born-dead" pups, pup weights ↑ Early pup deaths (at 100 ppm), ↓ weaning index
Siglin, 2000a	Rat (SD) (8/group)	gavage	$NiSO_4 \cdot 6H_2O$	0, 10, 20, 30, 50, 75 mg/kg/day 2 weeks prior to mating through 1 generation of animals	0, 2.2, 4.5, 6.7, 11.2, 16.7 [d]	No effect on mating, fertility parameters ↑ Dead pups, ↓ mean litter size at 75 mg/kg/day (perhaps at all doses) No effect on pup growth or survival ↑ Post-implantation loss at ≥30 mg/kg/day

Reference	Species/strain (N)	Route	Test material	Reported doses/duration	Nickel doses (mg Ni/kg/ day)	Effects
Siglin, 2000b	Rat (SD) (28/group)	gavage	$NiSO_4 \cdot 6H_2O$	0, 1, 2.5, 5 and 10 mg/kg/day 10 weeks prior to mating through 2 generations of animals	0, 0.22, 0.56, 1.1, 2.2 [d]	No effects on estrous cyclicity, sperm parameters, copulation and fertility indices, precoital intervals, gestation length, pup viability & growth, onset of sexual maturation No organ weights changes or histopathologic findings of the reproductive organs
Mice						
Rao et al., 2009	Mouse (Swiss albino) (10/group)	oral	$NiCl_2$	0, 8, 16 mg/kg/day $NiCl_2$ [e] 30 days	0, 3.6, 7.3 [f]	↓ Ovary absolute weights, but ↓ BWs also Ovary ↓ protein, GSH, ascorbic acid, SOD, CAT Ovary ↑ lipid peroxidation
Saini et al., 2014a	Mouse (Swiss albino) (10/group)	drinking water	$NiCl_2 \cdot 6H_2O$	0, 46, 92, 185 mg/kg/day Ni GD 0-5	0, 46, 92, 185	↓ Implantations, live fetuses (all doses) ↑ Resorptions, post-implantation losses ↓ Fetal weights, placental weights (top dose)
Saini et al., 2014b	Mouse (Swiss albino) (15/group)	drinking water	$NiCl_2 \cdot 6H_2O$	0, 46, 92, 185 mg/kg/day Ni GD 0-5, GD 6-13, or GD 14-18	0, 46, 92, 185	↓ Litter size (more prominent with pre-implantation dosing) ↓ Gestation index w/ pre-implantation dosing ↑ Pup mortality (more prominent with late gestation dosing) ↓ Early (before PND 4) & to weaning pup survival, ↓ pup weights
Cows						
O'Dell et al., 1970	Cows (5/group)	feed	$NiC0_3$	0, 50, 250 ppm Ni (0, 365, 1835 mg Ni/animal) 6 weeks	0, 0.46, 2.3 [g]	No effect on milk production, milk composition, or Ni concentration in milk

[a] Other groups received other elements in drinking water, including titanium and lead in rats or selenium, arsenic, lead, molybdenum and cadmium in mice.
[b] Calculated based on assumed rat body weight of 0.25 kg and assumed daily water consumption of 35 mL
[c] Some groups were with or without 0.3 ppm selenium.
[d] Calculated based on molecular weight ratio of Ni to $NiSO_4 \cdot 6H_2O$, assumed rat body weight of 0.25 kg and assumed daily water consumption of 35 mL
[e] Other dose groups were administered, vitamin E, $K_2Cr_2O_7$, and combinations of $NiCl_2$ and $K_2Cr_2O_7$ with and without vitamin E.
[f] Calculated based on molecular weight ratio of Ni to $NiCl_2$
[g] Calculated based on assumed dairy cow weight of 800 kg

Ovarian histopathology

Kong et al. (2014) is the only study that assessed the effects of nickel metal (administered by gavage as either nanoparticles or microparticles). This study reported no effect of nickel exposure on the mean weight of the ovary relative to body weight as well as no effects on mating or fertility. However, histopathologic alterations of the ovary were reported. These changes included increased vascular dilation and congestion; lymphocytosis; an increase in and cavitation of luteal cells; and infiltration of eosinophils and inflammatory cells into the ovary. Siglin (2000b) – the only other study to examine ovarian histology – found no alterations in association with nickel treatment.

Unfortunately, the photomicrographs provided by Kong et al. (2014) were very small and of poor quality, making it difficult for the reader to independently discern the reported findings. Additionally, the study authors failed to provide any context for their findings of the ovary in the discussion; thus, no mechanistic explanation for the reported findings was offered. It is also important to note that the study investigators did not report on the stage of estrus of the female rats at the time of sacrifice. This is an important omission, as estrous cyclicity changes in ovarian histology are common. Vacuolation of corpora luteal (CL) cells and an increase in vacuolated CL cells are common degenerative changes noted during proestrus (Dixon et al., 2014). At this stage, CL cells may also show increased mononuclear infiltrates (Dixon et al., 2014). Thus, some or all of the ovarian histologic changes observed in the nickel exposed animals in Kong et al. (2014) may be related to an increase in the number of animals undergoing proestrus at the time of sacrifice. Whether such a change is due to treatment is questionable as there was no effect on mating, fertility or precoital intervals in this and other studies (Smith et al., 1993; Käkelä et al., 1999; Siglin, 2000a,b). Siglin (2000b) also reported no effect of nickel on estrous cyclicity, but the doses may have been too low to be informative on this endpoint.

Because the reported ovarian changes may be an artifact related to the stage of estrus at which and because these changes were only reported in a single study involving oral exposure to animals, it is our opinion that these select data do not meet DARTIC's criteria for listing nickel metal nanoparticles or nickel compounds as a reproductive toxicant (OEHHA, 1993).

Hormone alterations

Kong et al. (2014) also reported changes in the serum concentrations of follicle stimulating hormone (FSH), luteinizing hormone (LH) and estradiol (E2) in female rats orally exposed to nickel metal nanoparticles and microparticles. Like changes in ovarian histology, however, alterations in serum levels of these hormones are cyclically related to the stage of estrus. Increases in both FSH and LH are associated with proestrus (Taya and Igarashi, 1973; Kannen, 1986). These increases, in turn, stimulate granulosa cells to synthesize and secrete E2 (Kannen, 1986). Thus, the alterations in serum hormones observed in the nickel-treated female rats – like the ovarian histological changes – may be related to a non-treatment-related increase in the number of animals undergoing proestrus at the time of sacrifice. Because of this and the fact that the finding was reported in a single study, it is our opinion that the Kong et al. (2014) data on female serum hormone alterations are not sufficient to meet DARTIC's criteria for listing of nickel metal or nickel compounds as a reproductive toxicant (OEHHA, 1993).

Effects on implantation

Saini et al. (2014a,b) reported in two separate mouse studies a reduction in the number of implantations with nickel exposure in the drinking water from gestational day (GD) 0 to GD 5. None of the other studies reviewed herein provide evidence of an adverse effect of nickel on the process of implantation. Siglin (2000a) reported a reduction in mean litter size with gavage exposure at 75 mg/kg/day in rats. However, no significant effect on implantation was seen; rather, post-implantation losses were observed. The definitive study (Siglin, 2000b) also saw no effect on implantation, but again, the doses may have been too low to be informative on this endpoint.

It is our opinion that the data on implantation are extremely limited, having been shown in only two studies conducted by the same group of study investigators and in a single species, the mouse. Therefore, it is our opinion that these data are insufficient to warrant the listing of nickel metal or nickel compounds as a reproductive toxicant.

Perinatal/early postnatal pup deaths

Multiple studies involving oral exposure of animals to nickel and nickel compounds reported increased perinatal and early pup mortality with treatment. These include:

- Kong et al. (2014), which reported a reduced "birth survival rate" at all doses of nickel nanoparticles and microparticles in rats;
- Schroeder and Michener (1971), which reported increased young rat pup deaths with 5 ppm (0.7 mg Ni/kg) nickel in drinking water;
- Smith et al. (1993), which reported increased litters with dead rat pups at birth and increased dead pups on postnatal day (PND) 21 with 250 ppm (31.6 mg Ni/kg) nickel in the drinking water (but not at lower concentrations);
- Käkelä et al. (1999), which reported a reduction in the percentage of rat pups born that survived until PND 4 (reduced viability index) at 100 ppm (14 mg Ni/kg) nickel in the drinking water (but not at lower concentrations);
- Siglin (2000a), which reported increased dead rat pups on PND 0 and reduced mean live litter size at 16.7 mg Ni/kg/day nickel (but not at lower gavage doses); and
- Saini et al. (2014b), which reported a reduced mean litter size and increased mouse pup mortality with nickel exposure at 92.25 mg Ni/kg/day nickel in the drinking water.

The reason for the inconsistency between the study by Schroeder and Michener (1971), which reported effects at a very low drinking water concentration of 5 ppm, and other drinking water studies (Smith et al., 1993; Käkelä et al., 1999) that reported no effects except at the highest drinking water concentrations is not clear. It should also be noted that some of these studies reported additional effects on pup survival to weaning (Kong et al., 2014; Smith et al., 1993; Käkelä et al., 1999; Saini et al., 2014b); however, the degree of response in the later lactation period (after PND 4) was less than that reported for perinatal/early postnatal mortality. Further, the finding was not reported in all studies (e.g., Siglin, 2000a).

It is our opinion that, unless there are specific data to show that the effect is mediated at the level of the maternal animal, perinatal/early postnatal pup death (and reduced survival to weaning) should be classified as a developmental effect. In the case of nickel, the limited

dataset available for our review does not show sufficient evidence that this response is mediated on the maternal animal instead of the offspring directly. While Smith et al. (1993) suggested a possible effect of nickel exposure on prolactin levels in the dam, a clear dose-response was not evident; further, it is not known if the degree of change in prolactin concentration would have been sufficient to result in a function change in milk production or maternal care. Finally, while the study by O'Dell et al. (1970) suggests no effect of nickel on milk production or composition, the study was conducted in cows (not rats), the doses administered were relatively small, the form of nickel used in the study ($NiCO_3$) is not soluble in water and its possible adherence to the feed is unknown. Thus, the oral absorption of the nickel in the only slightly acid rumen of the cow (pH 6-7) is questionable. Therefore, neither of these studies provide sufficient information to say whether the effect was likely to be maternally-mediated or not.

In summary, it is our opinion that the data on perinatal/early postnatal pup mortality (and pup survival to weaning) with oral nickel exposure to soluble nickel compounds in animals are sufficient to warrant listing of soluble nickel compounds as chemicals known to cause developmental toxicity. However, in the absence of other information, we believe this finding should not be considered an indicator of female reproductive toxicity. Rather, it is our opinion that these data support the classification of soluble nickel compounds based on developmental toxicity only. Because the data on nickel metal nanoparticles are from a single study, the results of which are sparsely reported and not clear based on questions regarding the statistical analyses presented, we do not believe that these data are sufficient to warrant classification of nickel metal nanoparticles as a developmental toxicant at this time.

Other findings

Rao et al. (2009) reported effects on a number of markers on oxidative stress in the ovary following subchronic exposure to $NiCl_2$ orally. It is important to note that these markers were not evaluated in any of the other female reproductive toxicity studies we were asked to evaluate. Further, they are not considered apical endpoints; therefore, their utility for addressing reproductive function is unclear. In the absence of additional mode of action (MOA) data that link these findings to specific adverse outcomes, they should not be used for hazard assessment.

Male Reproductive Toxicity

Ten (10) studies involving oral exposure of animals to nickel or nickel compounds and evaluation of potential male reproductive effects were reviewed; these are listed in Table 2. These studies are considered to be of moderate to low quality. Most were primarily mechanistic investigations that used few animals per group. Further, as will be discussed in greater detail below, the methods used in some of the studies for the fixation of tissues were likely poor, which adversely affects their reported outcomes. While the studies of Kong et al. (2014) and Siglin (2000b) included multiple dose groups, relatively large numbers of animals per group, and the incorporation of dosing regimens typically used for reproductive toxicity assessments, they also suffer from significant issues that affect their quality. Kong et al. (2014) evaluated non-standard endpoints not typically assessed in reproductive toxicity evaluations and suffers from methodological shortcomings. Additionally, as previously noted, the doses administered Siglin (2000b) were likely too low to be informative. Thus, as a whole, these studies are not particularly informative for assessing male reproductive toxicity.

Table 2. Select studies of oral nickel exposure on male reproductive endpoints.

Reference	Species/strain (N)	Route	Test materials	Reported doses/duration	Nickel doses (mg Ni/kg/day)	Effects
Rats						
Kong et al., 2014	Rat (SD) (10/group)	gavage	Ni nano-particles (NP) or micro-particles (MP)	NP: 0, 5, 15, 45 mg/kg/day MP: 45 mg/kg/day 10 weeks prior to mating through lactation	0, 5, 15, 45	No effect on mating or fertility ↑ testes, epididymis relative weights Seminiferous tubule histopath (epithelial shedding, disordered cells, apoptosis, cell death) Altered sperm motility parameters ↓ FSH, Test; ↑ LH
Schroeder and Michener, 1971	Rat (Long Evans) (5/group)	drinking water	Ni-soluble salt (specific form not reported)	5 ppm Weaning to 9 months of age and through 3 generations of rats	0, 0.7 [a]	↓ F3 litter size
Käkelä et al., 1999	Rat (Wistar) (6/group)	drinking water	$NiCl_2 \cdot 6H_2O$	30 ppm Ni [b] 4 or 6 weeks prior to mating	4.2 [a]	↓ Fertility, gestation index, litter size at weaning (no relation to duration) ↑ Early pup mortality (no relation to duration) Seminiferous tubule histopath (smaller tubules in middle of testes, ↓ basal spermatogonia at outer edge of tubules with 28-day, but not 42-day, exposure)
Obone et al., 1999	Rat (SD) (8/group per timepoint)	drinking water	$NiSO_4 \cdot 6H_2O$	0, 0.02, 0.05, 0.1% (0, 44.7, 111.75, 223.5 mg Ni/L) 13 weeks	0, 6.3, 15.6, 31.3 [a]	No effect on testes relative weights, markers of testicular damage; no testes histopathology

Reference	Species/strain (N)	Route	Test materials	Reported doses/duration	Nickel doses (mg Ni/kg/day)	Effects
Siglin, 2000a	Rat (SD) (8/group)	gavage	$NiSO_4 \cdot 6H_2O$	0, 10, 20, 30, 50, 75 mg/kg/day 2 weeks prior to mating through 1 generation of animals	0, 2.2, 4.5, 6.7, 11.2, 16.7 [c]	No effects on mating and fertility parameters
Siglin, 2000b	Rat (SD) (28/group)	gavage	$NiSO_4 \cdot 6H_2O$	0, 1, 2.5, 5 and 10 mg/kg/day 10 weeks prior to mating through 2 generations of animals	0, 0.22, 0.56, 1.1, 2.2 [c]	No effects on sperm parameters, copulation and fertility indices, precoital intervals, onset of sexual maturation No organ weight changes or histopathologic findings of the reproductive organs
Mice						
Pandey et al., 1999	Mice (Swiss albino) (20/group)	gavage	$NiSO_4$	0, 5, 10 mg/kg/day $NiSO_4$ 5 days/week for 5 weeks	0, 1.9, 3.8 [d]	↓ Testes, epididymis, seminal vesicle, prostate relative weights ↓ Sperm counts and motility, ↑ sperm abnormalities at 10 mg/kg/day Altered testicular damage markers At 10 mg/kg/day, testicular histopath (moderate congestion in peripheral region, atrophy of central seminiferous tubules w/increased intertubular spaces), cauda epididymides (regressed epithelium, cells vacuolated, anucleated, increased cytoplasmic granules), seminal vesicles (reduced size of vesicles) ↓ Fertility

Reference	Species/strain (N)	Route	Test materials	Reported doses/duration	Nickel doses (mg Ni/kg/day)	Effects
Pandey and Srivastava, 2000	Mice (strain not reported) (6/group)	oral (likely gavage)	$NiSO_4$	0, 5, 10, 20 mg/kg/day $NiSO_4$ 5 days/week for 5 weeks	0, 1.9, 3.8, 7.6 [d]	↓ Testes, epididymis, seminal vesicle, prostate relative weights (at 20 mg/kg/day) ↓ Sperm counts and motility at ≥10 mg/kg/day ↑ Sperm abnormalities
			$NiCl_2$	0, 5, 10, 20 mg/kg/day $NiCl_2$ 5 days/week for 5 weeks	0, 2.3, 4.5, 9.1 [e]	↓ testes, epididymis, seminal vesicle, prostate relative weights ↓ Sperm counts and motility at ≥10 mg/kg/day ↑ Sperm abnormalities
Panday and Singh, 2001	Mice (Swiss albino) (10/group)	oral (likely gavage)	$NiSO_4$	0, 20 mg/kg/day 5 days/week for 6 months	0, 7.6 [d]	No effect on testes abs weights No effect on testes histology ↓ Seminal vesicles abs weight, diameters, altered epithelium secretory activity
Toman et al., 2012	Mice (ICR) (5/group)	oral (given as pellets)	$NiCl_2$	0, 10 mg/kg/day 3, 6, 9 or 12 weeks	0, 4.5 [e]	No effect on testes relative weights Testicular histopath (degeneration of seminiferous epithelium, increased tubular lumen and empty spaces, reduced interstitial and germinal epithelium)

[a] Calculated based on assumed rat body weight of 0.25 kg and assumed daily water consumption of 35 mL
[b] Some groups were with or without 0.3 ppm selenium; no male control group was included.
[c] Calculated based on molecular weight ratio of Ni to $NiSO_4 \cdot 6H_2O$, assumed rat body weight of 0.25 kg and assumed daily water consumption of 35 mL
[d] Calculated based on molecular weight ratio of Ni to $NiSO_4$
[e] Calculated based on molecular weight ratio of Ni to $NiCl_2$

Hormone alterations

Kong et al. (2014) reported changes in the serum concentrations of FSH, LH, and testosterone (T) in male rats orally exposed to nickel metal. The changes in FSH and LH levels were different than those reported for female rats. However, Kong et al. (2014) do not report on when they took their serum samples and whether it was roughly the same time for all treatment groups or done in group order. This is an important omission, as circadian rhythms in the serum concentrations of FSH and LH have been reported for male rats (Taya and Igarashi, 1974). Thus, the alterations in serum hormones observed in the nickel-treated male rats may be related to the order in which the rats were sampled. Because of this and the fact that the finding was reported in a single study, it is our opinion that the Kong et al. (2014) data on male serum hormone alterations, like those for female hormone levels, are not sufficient to meet DARTIC's criteria for listing as a reproductive toxicant (OEHHA, 1993).

Organ weights

Seven (7) studies measured testis weights after oral exposure to nickel compounds. Kong et al. (2014) found increased relative epididymal and testicular weights in rats exposed by oral gavage to 45 mg Ni/kg/day. Obone et al (1999) and Siglin (2000b) reported no change in testicular weights in rats exposed to $NiSO_4 \cdot 6H_2O$ and Toman et al (2012) found no change in the relative testicular weights in mice exposed to $NiCl_2$ in their feed for up to 12 weeks. In contrast, 2 oral gavage studies by Pandey's group (Pandey et al., 1999; Pandey and Srivastava, 2000) found decreases in male reproductive organ weights (testes, epididymides, seminal vesicles and prostate gland) of mice exposed to either $NiSO_4$ or $NiCl_2$, whereas Pandey and Singh (2001) found no effect on testicular weight in mice gavaged with $NiSO_4$, although the weights of the seminal vesicles were decreased.

There is no clear pattern regarding species, route of administration, or form of nickel that aligns with the findings. Consequently, the database is inconsistent, and conclusions cannot be drawn about the effects of oral exposure of nickel on testicular weights. Thus, it is our opinion that these data are not sufficient to meet DARTIC's criteria for listing as a reproductive toxicant (OEHHA, 1993).

Testicular histopathology

Fixation of most male reproductive tissues (epididymides, seminal vesicles and prostate gland) and all female reproductive tissues (ovaries, oviducts, uteri) can be performed using neutral buffered formalin (NBF). The anatomy of the testis, however, is unique (Mescher, 2016). Due to the thick capsule surrounding the testis (tunica albuginea) and the rapid deterioration of testicular tissue, the testes must be processed using a rapid penetrating fixative such as modified Davidson's Fluid (mDF) or Bouin's solution. The best practices for testicular tissue preparation, including the recipe for mDF, are described in Latendresse et al. (2002). Briefly, the testes are dissected from the scrotum quickly but gently. At each of the poles of the testis, the tunica albuginea should be pierced ~5 times with a 30-gauge needle to a depth of 5-8 mm to enhance rapid penetration prior to immersion in mDF or Bouin's solution. After 48 hours, the testes should be removed from the initial fixative, rinsed with tap water and placed in NBF for storage prior to trimming and preparation for sectioning.

Testicular histopathology was evaluated in 7 studies. Three (3) studies found no effects (Pandey and Singh, 2001; Siglin, 2000b; Obone et al., 1999). Of these studies, all except Obone et al. (1999) used an appropriate fixation technique. Obone et al. (1999) provided no images to evaluate the integrity of the seminiferous epithelium because they found "no microscopic changes in any of the tissues examined."

Four (4) studies reported histological changes in the testes. Appropriate fixation methods were reported to have been used in the studies by Toman et al. (2012) and Käkelä et al. (1999). However, the description provided in Käkelä et al. (1999) – smaller tubules in the center of the testes – is consistent with incomplete penetration of the fixative. Further, the hypothesis posited of partial recovery with continued exposure does not appear plausible. The micrographs published in Pandey et al. (1999) are consistent with changes seen in testes that were improperly fixed with formalin. It is interesting to note that after the Pandey group changed to a fixation method that is now recommended (Bouin's fluid), they found no changes in the testes of treated mice, although they did report a change of columnar to cuboidal epithelium in the seminal vesicles. Such a change is seen under normal physiologic conditions as the secretory activity of a tissue changes. The micrographs published in Kong et al. (2014) are too small and at too low

of a magnification to enable an objective evaluation of their findings; additionally, they used the wrong fixative (paraformaldehyde), which could have led to artifacts. Toman et al. (2012) fed mice nominal doses of 10 mg $NiCl_2$/kg/day for 12 weeks. Micrographs of testicular tissue sections made after 9 and 12 weeks of exposure show degradative changes in the seminiferous tubules. It is not clear what the exact doses were in Toman et al. (2012) because $NiCl_2$ was incorporated into feed pellets and the form of the salt (anhydrous versus hydrate) was not reported. If the anhydrous form was used, the dose would have been 4.5 mg Ni/kg/day if the mice actually consumed the test agent at the nominal dose. If the hydrate was used, the dose of Ni would have been 2.5 mg Ni/kg/day.

Taken together, the data related to histopathology are inconsistent. There are numerous issues with the methods of some studies including improper fixation, and the results of several studies are conflicting showing either no change or potential degradative effects of Ni. It is not clear if the results are affected by the test species or route of exposure. Consequently, it is our opinion that conclusions cannot be drawn about the effects of nickel on testicular histology; further, these data should not be used as the basis for listing nickel as a reproductive toxicant.

Alterations in sperm parameters

Four (4) studies investigated the potential effects of exposure to nickel compounds on sperm motility. Siglin (2000b) found no effects of 10 mg/kg/day (2.2 mg Ni/kg/day), but the doses may have been too low to be informative on this endpoint. Pandey et al. (1999) and Pandey and Srivastava (2000) exposed male mice by oral gavage to 5, 10 or 20 mg $NiSO_4$ or $NiCl_2$ for 5 days per week for 5 weeks, at which time they evaluated sperm parameters. At $\geq$10 mg/kg/day for both compounds, they reported decreased sperm counts and motility as well as increased numbers of abnormal sperm. The difficulty is assessing the data from these papers arises from the lack of methodological details, such as the temperature of the diluent for the epididymal sperm and microscope stage, the numbers of sperm counted per field, the numbers of fields counted per sample, and whether the investigators were blinded to treatment. Because the assessments were performed manually, it is important to understand the conditions under which they were performed. Sperm motility is greatly influenced by temperature, and therefore, not only must fluctuations in temperature be avoided, but also the temperature range must be

narrow to keep sperm from all groups performing at their peak activity level. No information regarding this was available, leaving much doubt about the quality of the methods.

Kong et al. (2014) gavaged male rats with 15 or 45 mg Ni/kg/day for 10 weeks. The treatment regimen had no effect on mating behavior or fertility, but computer-assisted sperm analysis (CASA) was used to report on 9 parameters related to sperm motility, 3 of which differed between the 45 mg/kg/day group and control. CASA is the preferred method for assessing sperm motility; interpretation of sperm motility parameters is discussed in Seed et al. (1996). Typically, the percentages of motile sperm and progressively motile sperm are reported. Sperm motility is determined based on the number of sperm with an average path velocity above a certain pre-defined threshold. Progressive motility is defined based on the percentage of motile sperm with a linear index (often straight-line velocity) over a pre-determined threshold. While Kong et al. (2014) reports on these particular sperm parameters, they did not establish thresholds of significance for them. More importantly, average path velocity, straight line velocity, and straightness did not differ from control values. Thus, it is highly likely that the percentages of motile sperm and progressively motile sperm were unaffected by treatment. Further, two of the three affected parameters – beat cross frequency (measured in Hertz) and linearity (presented as a percent) – differed by only 1 Hertz or 1%, respectively, from control.

Taken as a whole, the data on sperm motility are sparse and insufficient to support a firm conclusion regarding the presence of absence of an effect due to nickel exposure. Thus, they should not be used as the basis for the listing of nickel as a reproductive toxicant.

Effects on fertility

Four (4) studies investigated various nickel compounds for male reproductive toxicity. Siglin (2000a,b) gavaged rats with $NiSO_4 \cdot 6H_2O$ at nominal doses up to 75 mg/kg/day starting 2 weeks prior to mating. No effects were reported on fertility or other reproductive indices or pup survival and growth. Käkelä et al. (1999) exposed male rats to 30 ppm $NiCl_2$ for 4 or 6 weeks prior to mating. They found a decrease in the fertility and gestation indices as well as decreased litter size in the 4-week exposure group, but no changes from control with $NiCl_2$ in the 6-week exposure group. Not only are the data from Käkelä et al. (1999) internally inconsistent, but

also, they must be viewed with caution because the group size was only 6 rats. The estimated intake of nickel was 4.2 mg/kg/day. Due to the small number of studies, internal inconsistency with regard to male reproductive effects reported by Käkelä et al. (1999), and lack of reported effects in Siglin (2000a) at a dose that was four times higher than that used by Käkelä et al. (1999), it is not possible to draw firm conclusions about the potential male reproductive toxicity of soluble nickel salts.

Kong et al. (2014) gavaged male rats with 45 mg Ni/kg/day as nanoparticles for 10 weeks prior to mating. No effects on male reproductive performance or behavior were noted. These findings need to be confirmed by others before drawing any conclusions about the absence (or presence) of the potential male reproductive toxicity due to metallic nickel in nanoparticle preparations.

As a whole, the body of data on male fertility are inconsistent and insufficient to warrant the listing of nickel or nickel compounds as a male reproductive toxicant.

Conclusions

Exponent scientists were requested to review a select set of 15 animal studies involving oral exposure to nickel or nickel compounds in order to provide an opinion on the strength of the data with particular focus on female and male reproductive effects. These included 5 studies relevant to female reproductive effects, 5 studies relevant to male reproductive effects, and 5 studies that provided data addressing potential reproductive effects in both sexes. Most of the studies involved administration of soluble nickel salts; one involved exposure to nickel metal nanoparticles. In our analysis, we found that the studies were generally of limited methodological and reporting quality.

Potential female reproductive effects of concern noted in these studies include effects on hormone alterations, ovarian histology, effects on implantation, and perinatal/early postnatal pup deaths (and reduced pup survival to weaning). Only the data on perinatal/early pup deaths, and to a lesser extent, pup survival to weaning are considered of sufficient strength to warrant the listing of soluble nickel compounds as chemicals known to cause reproductive toxicity. However, in the absence of information showing that these effects are mediated at the level of the maternal animal, it is our opinion that these data should be used to support the listing of soluble nickel compounds based on developmental toxicity only. No classification for female reproductive toxicity is warranted. Based on our survey of the injection studies cited for female reproductive toxicity in Tables C23-C26 of the OEHHA (2018) report, we conclude that these injection studies do not show additional data that would change our opinion regarding nickel's potential to cause female reproductive toxicity.

Potential male reproductive effects of concern addressed in the select set of studies reviewed herein include hormone alterations, effects on reproductive organ weights, testicular histopathology, alterations in sperm motility, and effects on fertility. For many of these parameters, the data were found to be highly contradictory across studies. The most compelling data are those on testicular histopathology. However, our analysis suggests that many of the studies reporting possible effects of nickel exposure on testicular histopathology are likely confounded by improper tissue fixation methods. This issue reduces the strength of these

studies for drawing conclusions regarding male reproductive toxicity. Based on this analysis, it is our opinion that, because of the conflicting nature of the findings reported and the methodological shortcoming regarding tissue fixation, the data reviewed herein are insufficient to warrant the listing of nickel and nickel compounds as a male reproductive toxicants. Our survey of the injection studies cited for male reproductive toxicity in Table D25 of the OEHHA (2018) report found multiple mechanistic investigations using few animals per group, a single treatment group, and extremely large (often 20 mg/kg/day) doses of $NiSO_4$. Many of these studies reported on mechanistic, rather than apical, endpoints. However, some also reported potential adverse effects on the male reproductive system similar to those discussed above. These studies, like the oral nickel studies reviewed herein, may suffer from methodological issues comparable to those examined above. Without further detailed analysis of these studies, we cannot draw a firm conclusion regarding the potential for nickel injection to cause adverse male reproductive effects. These studies, however, are not relevant to the expected routes for human exposure. Further, our evaluation of data from the oral studies do not support nickel's classification for male reproductive toxicity.

Limitations

The expert opinion expressed herein is made to a reasonable degree of scientific certainty. Exponent scientists reserve the right to supplement this report and to expand or modify the conclusions and findings based on review of additional, credible materials should they become available through additional work. This assessment may not adequately address the needs of other users of this report, and any re-use of this report or its findings and conclusions as presented herein are at the sole risk of the user.

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